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Amino Acid Signaling in *Saccharomyces cerevisiae*: a Permease-Like Sensor of External Amino Acids and F-Box Protein Grr1p Are Required for Transcriptional Induction of the *AGP1* Gene, Which Encodes a Broad-Specificity Amino Acid Permease

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The *SSY1* gene of *Saccharomyces cerevisiae* encodes a member of a large family of amino acid permeases. Compared to the 17 other proteins of this family, however, Ssy1p displays unusual structural features reminiscent of those distinguishing the Snf3p and Rgt2p glucose sensors from the other proteins of the sugar transporter family. We show here that *SSY1* is required for transcriptional induction, in response to multiple amino acids, of the *AGP1* gene encoding a low-affinity, broad-specificity amino acid permease. Total noninduction of the *AGP1* gene in the *ssy1*Δ mutant is not due to impaired incorporation of inducing amino acids. Conversely, *AGP1* is strongly induced by tryptophan in a mutant strain largely deficient in tryptophan uptake, but it remains unexpressed in a mutant that accumulates high levels of tryptophan endogenously. Induction of *AGP1* requires Uga35p(Dal81p/DurLp), a transcription factor of the Cys_6-Zn_2 family previously shown to participate in several nitrogen induction pathways. Induction of *AGP1* by amino acids also requires Grr1p, the F-box protein of the SCF^{Grr1} ubiquitin-protein ligase complex also required for transduction of the glucose signal generated by the Snf3p and Rgt2p glucose sensors. Systematic analysis of amino acid permease genes showed that Ssy1p is involved in transcriptional induction of at least five genes in addition to *AGP1*. Our results show that the amino acid permease homologue Ssy1p is a sensor of external amino acids, coupling availability of amino acids to transcriptional events. The essential role of Grr1p in this amino acid signaling pathway lends further support to the hypothesis that this protein participates in integrating nutrient availability with the cell cycle.

Yeast cells can selectively use the wide variety of nitrogenous compounds that they find in their rich natural environment. Some of these molecules can be directly used as ready-made metabolites. Many of them can also be catabolized to sustain the synthesis of glutamate and glutamine, the predominant nitrogen donors in biosynthetic reactions (20, 87). The synthesis of many enzymes and permeases involved in nitrogen metabolism and the activity of some of these proteins are tightly regulated according to the nitrogen source(s) available in the medium (20, 35, 45, 59, 87). It is generally assumed that these regulations are triggered solely by variations in the intracellular concentrations of specific metabolites. For instance, many enzymes involved in nitrogen anabolism are inhibited and/or their synthesis is repressed upon accumulation of the end or intermediate products of biosynthetic pathways (35, 45). Similarly, expression of most genes encoding amino acid biosynthetic enzymes is stimulated severalfold in response to starvation for any one of several amino acids (35, 45). Nitrogen repression (NR) is yet another example of regulation apparently triggered upon variation of the concentration of intracellular effectors (59). For instance, repression in the presence of NH_4^+ of at least some NR-sensitive genes is relieved in cells partially starved for glutamine due to a thermosensitive mutation in the glutamine synthetase *GLN1* gene (25, 87).

Whether yeast cells also possess regulatory systems responding specifically to the extracellular concentration of nitrogenous compounds has been studied very little to date. It seems reasonable, however, to speculate that such nitrogen sensors exist. For instance, two sensors of external glucose concentration (Snf3p and Rgt2p) have recently been discovered in yeast cells (67). These proteins are members of the sugar transporter superfamily (14, 15) and play a central role in the transcriptional regulation of the *HXT* genes encoding glucose transporters (55, 66, 67). Although Snf3p and Rgt2p show significant sequence similarity with hexose transporters, they seem unable to mediate glucose transport, or if they do, this activity is not sufficient to confer a measurable glucose uptake activity or to restore the ability to use glucose in a mutant lacking the six main glucose transporters (Hxt1, -2, -3, -4, -6, and -7) (55, 66, 73). Two other proteins of the sugar transport family, namely, the Rco3 regulator of conidiation in *Neurospora crassa* (58) and the Mst1 protein from the ectomycorrhiza *Amita muscaria* (64), may also serve as glucose sensors. Similarly, the *uhpC* gene of *Escherichia coli* encodes a protein highly similar in sequence to UhpT, a permease for several organophosphate compounds including glucose-6-phosphate (47). The UhpC protein seems unable to mediate uptake of glucose-6-phosphate and is involved, rather, in transcriptional induction of the *uhpT* permease gene in response to micromolar levels of external glucose-6-phosphate (48). Some cell surface proteins that effectively mediate transmembrane solute transport also

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
23344c	*MAT*α *ura3*	LPCGL[a]
23346c	*MAT***a** *ura3*	LPCGL
32501a	*MAT*α *ura3*	This study
32501b	*MAT*α *gap1*Δ::*kanMX2 ura3*	This study
32501c	*MAT***a** *ssy1*Δ::*kanMX2 ura3*	This study
32501d	*MAT***a** *gap1*Δ::*kanMX2 ssy1*Δ::*kanMX2 ura3*	This study
30629c	*MAT***a** *gap1*Δ::*kanMX2 ura3*	This study
30633c	*MAT***a** *gap1*Δ::*kanMX2 agp1*Δ::*kanMX2 ura3*	This study
32502b	*MAT*α *gap1*Δ::*kanMX2 agp1*Δ::*kanMX2 ssy1*Δ::*kanMX2 ura3*	This study
30622a	*MAT*α *gap1-92 agp1-1 ura3*	36a
30701a	*MAT***a** *aro80*Δ::*kanMX2 ura3*	36a
26247b	*MAT***a** *trp2*^{fbr}	82
RE1	*MAT***a** *trp2*^{fbr} *ura3*Δ::*kanMX2*	This study
JA115	*MAT*α *grr1*Δ::*kanMX2 ura3*	This study
CD17	*MAT*α *uga35*Δ::*TYR1 tyr1 ura3*	21

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seem to have a regulatory function. For instance, it was recently shown that Mep2p, a high-affinity NH_4^+ transporter (61), is essential to diploid-cell differentiation into a filamentous, pseudohyphal growth when the sole nitrogen source is NH_4^+ at a low concentration. This suggests that this transporter also acts as a sensor of low levels of extracellular NH_4^+ (56). These studies raise the possibility that yet other transporters or transporter homologs act as sensors of external compounds in addition to (or instead of) mediating their uptake across the plasma membrane. In fact, transmembrane solute transporters, by their location, diversity, and ability to recognize a wide variety of exogenous compounds with different specificities and affinities, seem ideally "qualified" to serve as sensors of external nutrients.

It was recently reported that the amino acid permease homologue encoded by the *YDR160w*/*SSY1* gene is required for transcriptional induction by leucine of the amino acid permease genes, *BAP2*, *TAT1*, and *BAP3*, and of the peptide transporter, *PTR2*. Induction of *BAP2* by L- or D-leucine also occurs in a strain largely deficient in L- and D-leucine transport, suggesting that Ssy1p mediates transcriptional induction of permease genes in response to external leucine (24). We report here the results of an independent approach indicating that this amino acid permease homologue acts as a sensor of multiple external amino acids. This sensor is required for transcriptional induction of at least six amino acid permease genes. We show that one of these genes, *AGP1*, encodes a wide-specificity amino acid permease induced by all amino acids except proline. Together with the general amino acid permease (Gap1p), this permease plays a major role in amino acid utilization. We show that induction of *AGP1* occurs in response to extracellular rather than intracellular amino acids. This induction requires the Cys_6-Zn_2 transcription factor encoded by the *UGA35*(*DAL81*/*DURL*) gene. It also requires the F-box protein Grr1p involved in cell cycle regulation and in Snf3p- and Rgt2p-mediated glucose sensing.

MATERIALS AND METHODS

Strains, growth conditions, and methods. The *Saccharomyces cerevisiae* strains used in this study are all isogenic with the wild-type Σ1278b (12) except for the mutations mentioned (Table 1). Cells were grown in a minimal buffered (pH 6.1) medium with 3% glucose as the carbon source (41). To this medium, urea (5 mM), proline (5 mM), $(NH_4)_2SO_4$ (10 mM), amino acids (1 to 10 mM), or combinations of these compounds were added as a source(s) of nitrogen. Assays for resistance to toxic amino acid analogues were carried out on plates with $(NH_4)_2SO_4$ (10 mM) as the sole nitrogen source. Analogue concentrations were as follows: 20 μg/ml, β-(2-thienyl)-DL-alanine; 20 μg/ml, *p*-fluoro-DL-phenylalanine; 20 μg/ml, D,L-ethionine; 500 μg/ml, 6-fluoro-tryptophan; and 1 mg/ml, hydroxy-tryptophan. All procedures for manipulating DNA were standard ones (6, 74). The *E. coli* strain used was JM109.

Construction of *ssy1*Δ, *agp1*Δ, *gap1*Δ, and *grr1*Δ deletion strains. The *ssy1*Δ, *agp1*Δ, *grr1*Δ, and *gap1*Δ null mutations were constructed by the PCR-based gene deletion method (86). The DNA segments used to introduce these mutations were generated by using the *kanMX2* gene from plasmid pFA6a-kanMX2 as a template and the following PCR primers: *ssy1*Δ:*kanMX2*, 5′-CTCTAGGGGAAAAAAGGAAACAGGCGTGTGATAAGAGGCCGCGGCCGCCAGCTGAAGCTTCGTACGC-3′ and 5′-CAGTTACCCGCACAATCTAGTGCGTAAAGCAGTGTCAATAGCGGCCGCATAGGCCACTAGTGGATCTG-3′; *agp1*Δ:*kanMX2*, 5′-CCAGAAGGCAACGACCCTTTTCCAATAAGGTCCGTTCCGCGGCCGC GCATAGGCCACTAGTGGATCTG-3′ and 5′-TCGTCGTCGAAGTCTCTATACGAACTGAAAGACTTGGCGGCCGCCAGCTGAAGCTTCGTACGA-3′; *gap1*Δ:*kanMX2*, 5′-CTATCAGGCAGCCTCACTAATCTACCCATTGACCTCATGCGCGGCCGCCAGCTGAAGCTTCGTACGC-3′ and 5′-GAAGCTCACACAGATTAGTTTTCATCTCGCTGTCTACTAAGCGGCCGCATAGGCCACTAGTGGATCTG-3′; and *grr1*Δ:*kanMX2*, 5′-ATGGATCAGGATAACAACAACCACAATGACAGCAATAGGCTGCACCCATTCGTACGCTGCAGGTCGAC-3′ and 5′-GGGCGTTCCTGATGCTTCATCCATTTGAGAATCAATGGCAGTGTCAGGCGCATAGGCCACTAGTGGATCTG-3′. The yeast strain 23344c was transformed with the PCR fragments by the lithium method (39) as described previously (30). Transformants were selected on complete medium containing 200 μg of G418 (Geneticin; Gibco BRL) per ml.

Plasmids. The YCp*ARO9-lacZ* plasmid has been described (36). The YCp*AGP1-lacZ* plasmid was constructed by inserting into the *Bam*HI- and *Hin*dIII-cleaved YCpAJ152 plasmid (4) a 996-bp DNA fragment flanked by *Hin*dIII and *Bam*HI restriction sites and spanning the five first codons of *AGP1* plus 979 bp of upstream sequences. This DNA fragment was obtained by PCR with, as a template, plasmid p16.2 bearing the *AGP1* gene cloned from strain Σ1278b (36a) and the following PCR primers: 5′-CCGAAGCTTCCTCAACCTACCATGGCAAAC-3′ and 5′-CGCGGATCCGACTTCGACGACGACATTGT-3′. The accuracy of the PCR-amplified fragment was checked by sequencing.

Enzyme and permease assays. All permease and enzyme assays were performed on cells that reached the state of balanced growth. Incorporation of ^{14}C-labeled amino acids (Amersham) was measured as previously described (33). β-Galactosidase activities were measured as described earlier (4) and are expressed in nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Protein concentrations were measured with the Folin reagent (57) and, as the standard, bovine serum albumin.

Measurements of intracellular tryptophan concentration. Cells having reached the state of balanced growth were collected by filtration (Millipore 0.45-μm-pore-size filters) and washed four times with ice water. Cells were immediately resuspended in 5 ml of 5% trichloroacetic acid and incubated at 0°C for 10 min with several inversions of the tubes. The extracts were harvested after filtration (Millipore 0.45-μm-pore-size filters) and stored at −20°C. Tryptophan concentrations were determined by high-pressure liquid chromatography with electrochemical detection as previously described (29).

RNA analysis by RT-PCR. Total yeast cell RNA was prepared by using the RNeasy Mini-Kit (Qiagen) as recommended by the manufacturer. RNA preparations were treated with DNase (Boehringer) for 1 h at 37°C and washed with RNeasy mini spin columns as described by the manufacturer (Qiagen). A PCR test was performed on each RNA preparation to make sure it was DNA-free. Reverse transcriptase (RT)-PCRs were performed by using the Titan One Tube RT-PCR Kit (Boehringer Mannheim) and a Tecne (Cambridge) thermocycler. The samples were first incubated at 55°C for 30 min (for reverse transcription) and then as follows for thermocycling: 94°C for 2 min (1 time); then 94°C for 30 s, 52°C for 30 s, and 68°C for 75 s, plus 5 s for each cycle starting at cycle 11 (25 times); and then 68°C for 7 min (1 time). The PCR primers were chosen so that they had similar melting temperatures and generated PCR fragments of similar sizes. Their sequences were as follows: *ACT1*, 5′-GACTCCTACGTTGGTGATGA-3′ and 5′-CTGGAGGAGCAATGATCTTG-3′; *GAP1*, 5′-ATCGGTACTGGTCTGCTGGT-3′ and 5′-TCTACGGATTCACTGGCAGC-3′; *AGP1*, 5′-TCTTACGTCGGCTATCTCAC-3′ and 5′-GATGCAACAGCAATGACATA-3′; *GNP1*, 5′-TGGTCACTGCATCCATGACT-3′ and 5′-GAGGCACAGAATGCAATGAC-3′; *BAP2*, 5′-TCGAGACGTACTTCATGATC-3′ and 5′-TCAGTCTTGGACCAGCATAC-3′; *TAT1*, 5′-GTCACTTAGTCATGATCAGT-3′ and 5′-ATGTGATGCAACAGCAATGA-3′; *TAT2*, 5′-ACCGTACAGTACTGGAACTC-3′ and 5′-CTGATATGTGACAGGTTGAT-3′; *BAP3*, 5′-ATCGGTTACGTTATGGTGTC-3′ and 5′-GCTGCCAAGACATATGGTGA-3′; *YNL270c*, 5′-GACAGAAGCAGTGCCTCTAG-3′ and 5′-CACCTCTGGTCACGTTAGAC-3′; *YBR132c*, 5′-CATTACTGTGTCTACAGCGG-3′ and 5′-AGTGTAAGCGTTACCAGCAG-3′; *YPL274c*, 5′-TGTCAGTAGGTTCATAGATG-3′ and 5′-GTCCATGTAGGAACATACCG-3′; *YLL061w*, 5′-TATCAAGATGACCGCATTCA-3′ and 5′-ATCACTAGCGTCCGGACCTG-3′; *YFL055w*, 5′-ATAGCGATGCACTGCCTGCA-3′ and 5′-TGCAGCTCCAACGCTCACAT-3′; *MUP1*, 5′-TCTGAATGTCAAGATTGGTC-3′ and 5′-GTAAGGAGCAATAATCAGGT-3′; and *MUP3*, 5′-ATAACCATCCATCGATACCA-3′ and 5′-CACGGATGATTCGTGGTCCA-3′.

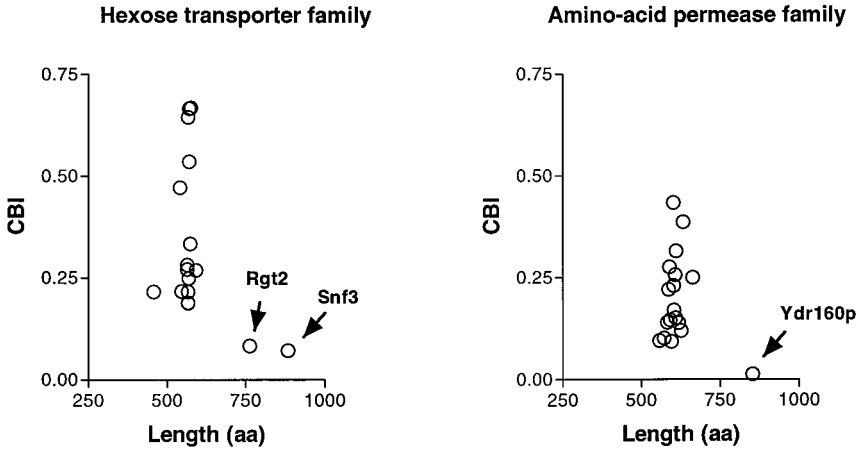

FIG. 1. The yeast Ydr160p protein of the amino acid permease family displays unusual features resembling those of the Snf3p and Rgt2p glucose sensors. The CBI values (13) of the genes coding for the 17 Hxt proteins of the hexose transporter family, the glucose sensors Snf3p and Rgt2p (left panel), and the 18 proteins of the amino acid permease family (right panel) are plotted against the number of amino acid residues present in the proteins.

RESULTS

Ydr160p, an amino acid permease homologue, displays unusual structural features. Complete sequencing of the yeast genome has led to the discovery of many new genes encoding putative transmembrane solute transporters (3). The largest class of yeast transport proteins is the sugar transporter family, which includes 7 functionally characterized hexose transporters (Hxt1, -2, -3, -4, -6, and -7 and Gal2), 10 homologous proteins of still unknown function, and 17 more distantly related proteins, some of which have been functionally characterized (15, 53). It is now accepted that two proteins of this family, Snf3p and Rgt2p, ensure a regulatory rather than a catabolic function, i.e., they act as sensors of external glucose concentration (55, 67). These two proteins display features that clearly distinguish them from the Hxt transporters: they are expressed to much lower levels and their C-terminal cytosolic tail is much longer (14, 19, 67). These properties are obvious when the codon bias index (CBI [13]) values of the genes coding for Snf3p, Rgt2p, and the Hxt proteins are plotted against the amino acid chain lengths of these proteins (Fig. 1). We have applied the same plot representation to all other families of yeast transport proteins extracted by computational analysis (3). Analysis of the output data concerning the 18 proteins of the amino acid permease family revealed that one member of this family, namely, the *YDR160w* gene product (42), stands out from the others because of its unusually low CBI (0.013) and much larger size (852 amino acids) (Fig. 1). Like the other proteins of this family, Ydr160p consists of a central hydrophobic core of 12 predicted transmembrane (TM) domains flanked by N-terminal and C-terminal hydrophilic regions. The hydrophilic N terminus of Ydr160p (281 residues), however, is unusually large compared to those of the other members of the amino acid permease family (Fig. 2). Furthermore, several regions connecting TM domains and predicted to be extracellular are larger in Ydr160p than in classical amino acid permeases (Fig. 2). Finally, Ydr160p is more distantly related in sequence to the other members of the amino acid permease family (Fig. 2). The unusual structural features of Ydr160p are reminiscent of those distinguishing the Snf3p and Rgt2p glucose sensors from the other proteins of the sugar transporter family (14, 19, 67). On the basis of these criteria, we hypothesized that Ydr160p might perform a function different from that of classical amino acid permeases. For instance, just as Snf3p and Rgt2p play a determining role in regulating glucose transport, Ydr160p might be involved in regulating amino acid transport.

Ydr160p-Ssy1p is required for induction of amino acid permeases. As a first step in the functional analysis of the *YDR160w* gene, we isolated a yeast strain with a complete deletion of the corresponding coding region (see Materials and Methods). The deletion mutant was viable on both rich and minimal glucose medium. The *ydr160*Δ mutant displayed no clear growth defect on any of the amino acids that can be used as the sole nitrogen source (data not shown). We then compared the sensitivities of the wild-type and *ydr160*Δ strains to several toxic amino acid analogues. These experiments showed that lack of the *YDR160w* gene confers resistance to the phenylalanine analogues β-(2-thienyl)-DL-alanine and *p*-fluoro-DL-phenylalanine, to the methionine analogue D,L-ethionine, and to the tryptophan analogues 6-fluoro-tryptophan and hydroxy-tryptophan (data not shown). Resistance to several toxic amino acid analogues is a property shared by *apf* mutants deficient in the uptake of multiple amino acids (32). The *YDR160w* gene proved to be nonallelic with six previously isolated *apf* complementation groups and was initially called *APF7* for amino acid permeability factor 7. In the course of preparation of this study, it was reported that the *SSY1* gene originally identified on a genetic basis (46) is identical to *YDR160w* (24). Hence, the *SSY1* nomenclature will hereafter be adopted.

The activity of the general amino acid permease (Gap1p) was unaffected in the *ssy1*Δ mutant growing on urea or proline as the sole nitrogen source, i.e., under conditions where Gap1p is normally most active (not shown). This observation prompted us to examine the effect of deleting *SSY1* in a *gap1*Δ mutant. We first compared growth of the *gap1*Δ and *gap1*Δ *ssy1*Δ strains on low (1 mM) and high (10 mM) concentrations of several amino acids, each used as the sole nitrogen source (Fig. 3). Deletion of the *SSY1* gene in the *gap1*Δ strain dramatically reduced growth on low concentrations of isoleucine, leucine, valine, methionine, phenylalanine, tyrosine, tryptophan and, to a lesser extent, threonine (Fig. 3). Growth of the *SSY1*-deleted strain returned to normal on leucine, valine, and threonine when the amino acid concentration was increased to 10 mM. At this higher concentration, a clear growth defect due to lack of Ssy1p was still visible on isoleucine, phenylalanine, tyrosine, tryptophan and, to a lesser extent, methionine.

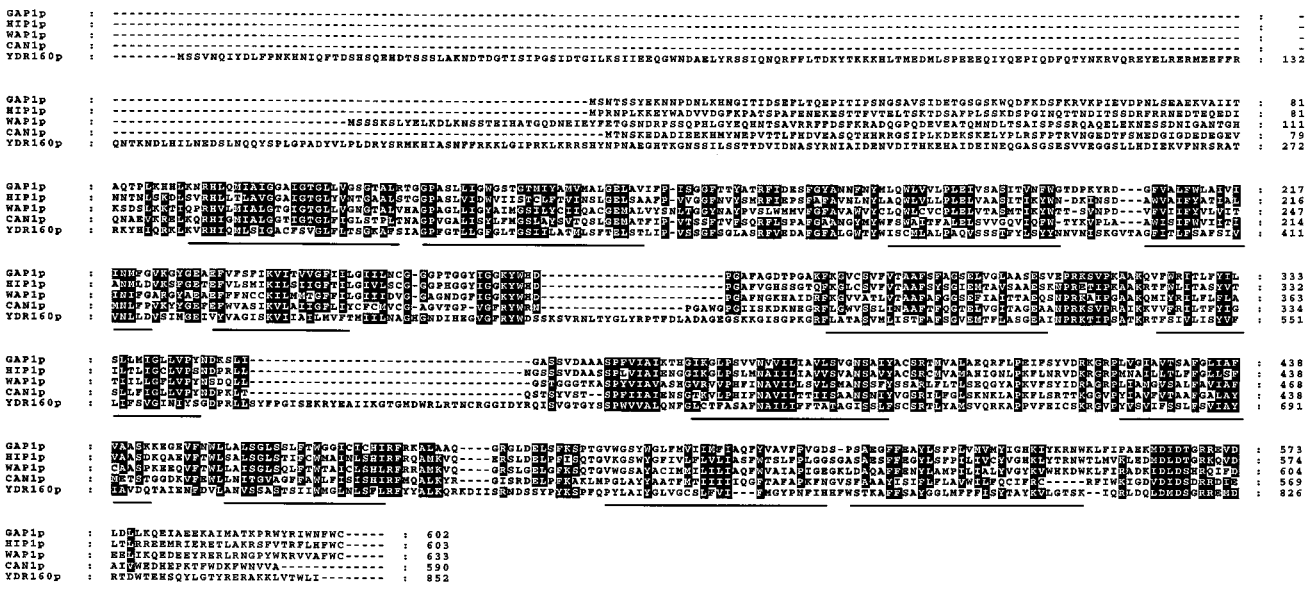

FIG. 2. The Ydr160-Ssy1p protein displays unusual structural features compared to other members of the amino acid permease family. The amino acid sequences of the amino acid permeases Gap1p (43), Hip1p (79), Agp1p-Wap1p (65), and Ydr160p-Sys1p (42) were aligned by using the PILEUP program (23). Identical and conserved residues are indicated in black boxes. The transmembrane segments predicted by using the TMAP algorithm (72) are underlined.

The effects of deleting *SSY1* in the *gap1*Δ strain largely overlap with those produced in the same genetic background by the *wap1*Δ/*agp1*Δ mutation (Fig. 3). *WAP1*, a gene originally discovered during systematic sequencing of chromosome III (*YCL025c*), encodes a member of the amino acid permease family (65). The *WAP1* gene (*WAP1* for wide-specificity amino acid permease 1) was also isolated in our laboratory as part of a study focusing on induction by aromatic amino acids of the *ARO9* gene encoding aromatic aminotransferase II. In that study, among mutants displaying reduced induction of an *ARO9-lacZ* fusion gene, one turned out to bear two mutations: one in the *GAP1* gene and another in the *WAP1* gene. In this *gap1–92 wap-1-1* mutant strain, induction of *ARO9-lacZ* is reduced severalfold compared to the wild type, an effect most likely due to partial inducer exclusion (36a). As this study was being prepared, a functional and expression analysis of the *YCL025c/WAP1* gene was reported and the gene was named *AGP1* (for asparagine and glutamine permease) (76). Hence, the *AGP1* nomenclature will hereafter be used here.

Deletion of the *AGP1* gene in the wild-type strain did not affect amino acid utilization (data not shown), but in the *gap1*Δ strain it produced phenotypes similar to those of the *gap1*Δ *ssy1*Δ mutant, except on valine (1 mM), methionine (1 to 10 mM), phenylalanine (10 mM), and tryptophan (10 mM), where growth of the *gap1*Δ *ssy1*Δ strain was more strongly affected than that of the *gap1*Δ *agp1*Δ strain (Fig. 3). These results are consistent with *SSY1* being needed for the function of the Agp1p permease, but since the growth deficiency caused by the *ssy1*Δ mutation is broader than that caused by the *agp1*Δ mutation, at least one additional amino acid permease is likely affected by the *ssy1*Δ deletion. The fact that the *ssy1*Δ strain is resistant to five toxic amino acid analogues and that the *agp1*Δ strain is sensitive to them (not shown) is also consistent with Ssy1p affecting other amino acid permeases in addition to Agp1p.

These assumptions were confirmed by amino acid uptake assays with cells growing on urea as the sole nitrogen source. Representative data obtained with the amino acids leucine, isoleucine, phenylalanine, and tyrosine are shown in Fig. 4. All four amino acids were immediately incorporated into *gap1*Δ cells at a relatively low rate, but uptake rapidly accelerated, a behavior typically observed in the case of permeases induced in the presence of their own substrates (4). In the *gap1*Δ *agp1*Δ double mutant, the inducible uptake of each amino acid was largely, though not entirely, suppressed (Fig. 4). Thus, the inducible leucine, isoleucine, phenylalanine, and tyrosine uptake activities displayed by *gap1*Δ cells growing on minimal urea medium are largely attributable to the product of the *AGP1* gene. The fact that *gap1*Δ *agp1*Δ cells still display residual inducible uptake activity suggests that each amino acid is incorporated by at least one additional inducible permease. In the *gap1*Δ *ssy1*Δ mutant, finally, all four amino acids were incorporated at a low and apparently constant rate, indicating that Agp1p and the additional permease(s) normally induced in response to leucine, isoleucine, phenylalanine, and tyrosine are inactive in the *ssy1*Δ strain. These results are consistent with the growth test data and show that Ssy1p is required for the activity of at least two inducible amino acid permeases, one being Agp1p.

Ssy1p is required for transcriptional induction of the *AGP1* gene in response to multiple amino acids. A DNA fragment composed of the first codons of the *AGP1* gene preceded by its promoter region was fused in frame with the *lacZ* reporter gene in a low-copy-number plasmid. Wild-type cells transformed with this *AGP1-lacZ*-bearing plasmid were grown on minimal medium containing urea, urea-leucine, urea-isoleucine, urea-phenylalanine, or urea-tyrosine as the sole nitrogen source(s). β-Galactosidase assays in extracts of steady-state growing cells (Table 2) clearly showed that *AGP1* is not expressed on urea medium but that its transcription is markedly induced in the presence of each of the four amino acids (lines 1 to 5). In the *ssy1*Δ strain, in contrast, the *AGP1-lacZ* gene remained uninduced (lines 1 to 5). We then tested the influence of other amino acids on expression of the *AGP1-lacZ* gene. Remarkably, many other amino acids induced transcription of the *AGP1-lacZ* gene, and in all cases induction was

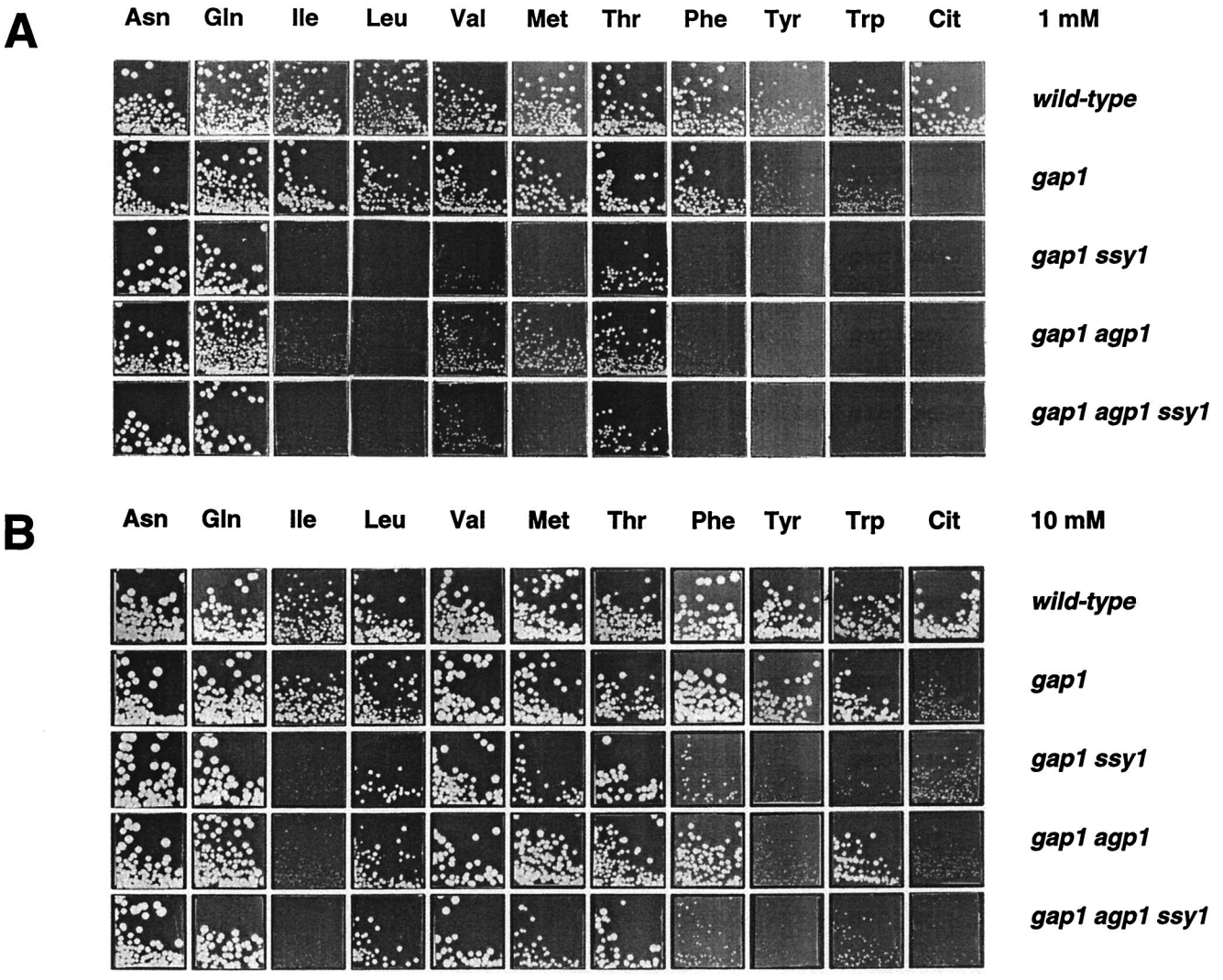

FIG. 3. Deletion of the *SSY1* or the *AGP1* gene affects the utilization of several amino acids in cells lacking the general amino acid permease (Gap1p). Cells were spread on minimal medium with the indicated amino acid at the final concentration of 1 mM (A) or 10 mM (B) as the sole nitrogen source. The strains were 23344c (*ura3*), 32501b (*gap1*Δ *ura3*), 32501d (*gap1*Δ *ssy1*Δ *ura3*), 30633c (*gap1*Δ *agp1*Δ *ura3*), and 32502b (*gap1*Δ *ssy1*Δ *agp1*Δ *ura3*).

abolished in the *ssy1*Δ strain (lines 6 to 23). The level of induction in the wild type, however, varied strongly according to the amino acid tested. The highest induction levels were produced by leucine, isoleucine, phenylalanine, tyrosine, tryptophan, threonine, and methionine, i.e., amino acids on which growth of the *gap1*Δ strain was most affected after deletion of the *SSY1* gene. Intermediate levels of induction were obtained with valine, citrulline, cysteine, alanine, and serine; and still lower levels were obtained with lysine, histidine, glutamate, glutamine, glycine, aspartate, and asparagine. 4-Aminobutyrate (GABA), arginine, and ornithine were very poor inducers, and the presence of proline or certain other nitrogenous compounds that can serve as nitrogen sources (allantoate, allantoin, cytosine, and adenine) had no influence on *AGP1-lacZ* expression. In conclusion, transcription of the *AGP1* gene is induced by many amino acids (a notable exception being proline), and this induction requires a functional *SSY1* product.

***AGP1* encodes a broad-specificity, low-affinity amino acid permease.** That the *AGP1* gene is induced to various degrees by multiple amino acids suggests that the amino acid permease encoded by this gene has a broad substrate specificity. In amino acid uptake experiments (Fig. 4), induction of *AGP1* clearly led to markedly increased uptake of leucine, isoleucine, phenylalanine, and tyrosine. These uptake assays in fact show that, apart from the general amino acid permease, Agp1p is the main entry pathway for these four amino acids (used at 0.1 mM concentrations) in cells growing under conditions of nitrogen derepression. To further explore the substrate specificity range of the Agp1p permease, we compared in the *gap1*Δ and *gap1*Δ *agp1*Δ strains the initial uptake rates of several amino acids present at a 0.1 mM concentration (Table 3). To induce *AGP1* expression, we grew the strains on minimal urea medium supplemented with citrulline (0.5%). We found that citrulline used at this concentration does not significantly interfere with Agp1p-mediated uptake of amino acids (inhibition was ≤10%), i.e., it behaves like a gratuitous inducer of *AGP1* expression. In the *gap1*Δ strain grown under these conditions, Agp1p proved responsible for a significant portion of the initial uptake of many amino acids, including leucine (96%), isoleucine (86%), tyrosine (83%), valine (82%), phenylalanine

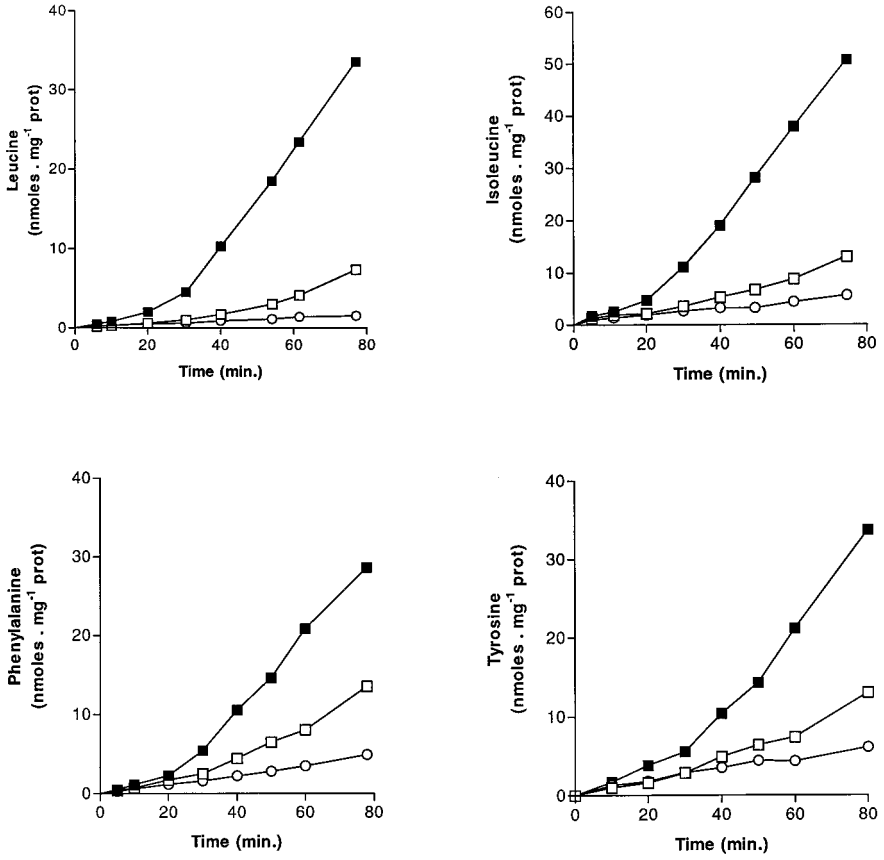

FIG. 4. Deletion of the *SSY1* or *AGP1* gene alters incorporation of amino acids. The time course of ^{14}C-labeled leucine, isoleucine, phenylalanine, and tyrosine (initial concentration, 0.1 mM) accumulation measured in cells growing on minimal medium with urea as the sole nitrogen source is shown. The strains were 30629c (*gap1*Δ *ura3*) (■), 30633c (*gap1*Δ *agp1*Δ *ura3*) (□) and 32501d (*gap1*Δ *ssy1*Δ *ura3*) (○).

(78%), threonine (77%), methionine (68%), glutamine (64%), serine (62%), alanine (60%), histidine (54%), glycine (49%), and asparagine (25%). It contributed negligibly to the uptake of tryptophan, proline, arginine, aspartate, glutamate, and lysine (0 to 15%). Hence, most amino acids inducing high-level expression of *AGP1* appear to be substrates of Agp1p. The fact that deletion of *AGP1* in a *gap1*Δ mutant alters growth on only some of the Agp1p substrates is likely due to the existence of other permeases that can compensate for the lack of Agp1p. For instance, a permease able to transport glutamine (Gnp1p) has been described (90). Although we could not detect any significant contribution of Agp1p to the uptake of tryptophan, proline, arginine, lysine, glutamate, aspartate, or citrulline used at concentrations of up to 0.1 mM, at least some of these amino acids are likely incorporated via Agp1p when present at a higher concentration. For instance, Agp1p is required for a *gap1* mutant to grow on tryptophan at a 1 mM concentration (Fig. 3). Agp1p is a relatively low-affinity permease, as judged by the kinetic parameters of Agp1p-mediated leucine uptake (K_m = 0.16 mM; V_{max} = 100 nmol · min^{-1} · mg of protein^{-1}), isoleucine (K_m = 0.6 mM; V_{max} = 175 nmol · min^{-1} · mg of protein^{-1}) and phenylalanine (K_m = 0.6 mM; V_{max} = 60 nmol · min^{-1} · mg of protein^{-1}).

Nonexpression of the *AGP1* gene in the *ssy1*Δ mutant is not due to inducer exclusion. As the Ssy1p protein is a member of the amino acid permease family, one might argue that noninduction of *AGP1* in the *ssy1*Δ strain is due to insufficient incorporation of the inducing amino acids. To test this possibility, we grew the wild type and the *ssy1*Δ strain, both harboring the *AGP1-lacZ* fusion gene, on minimal urea medium. After addition of ^{14}C-labeled leucine, isoleucine, tyrosine, or phenylalanine, culture samples were withdrawn at intervals and used in parallel experiments to assay β-galactosidase activity and the incorporation of ^{14}C-labeled amino acids (Fig. 5). *AGP1-lacZ* was induced in response to each amino acid tested, and this induction was abolished in the *ssy1*Δ strain. The *ssy1*Δ strain incorporated all four amino acids at a high rate, so noninduction of the *AGP1-lacZ* gene in the *ssy1*Δ strain is not due to inducer exclusion. In fact, the *ssy1*Δ strain even accumulated greater amounts of ^{14}C-labeled amino acids than did the wild type, an effect not further investigated here. Whatever might cause this effect, it is clear that noninduction of the *AGP1* gene in the *ssy1*Δ mutant is not due to reduced uptake of amino acid inducers. Rather, Ssy1p behaves like a regulatory factor essential to transcriptional induction of the *AGP1* gene in response to multiple amino acids.

Ssy1p mediates induction of the *AGP1* gene in response to external amino acids. In a next set of experiments we addressed the question of whether Ssy1p-dependent expression of the *AGP1* gene is induced in response to intracellular or extracellular amino acids. For comparison, we extended this analysis to the *ARO9* gene inducible by aromatic amino acids. Unlike *AGP1*, induction of *ARO9* by tryptophan is normal in the *ssy1*Δ mutant but is abrogated in cells deleted of *ARO80*, a gene encoding a transcription factor of the Cys_6-Zn_2 family. Conversely, induction of *AGP1* by aromatic amino acids is

TABLE 2. The Ssy1 protein is required for transcriptional induction of the *AGP1* gene in response to multiple amino acids[a]

Line no.	Nitrogen sources	*AGP1-lacZ* β-galactosidase activity (nmol · min^{-1} · mg of protein^{-1})	
		Wild type	*ssy1*Δ
1	Urea	≤2	≤2
2	Urea + Leu	713	≤2
3	Urea + Ile	1,069	≤2
4	Urea + Phe	943	≤2
5	Urea + Tyr	903	≤2
6	Urea + Trp	1,246	≤2
7	Urea + Thr	871	≤2
8	Urea + Met	825	≤2
9	Urea + Val	421	≤2
10	Urea + citrulline	294	≤2
11	Urea + Cys	241	≤2
12	Urea + Ala	198	≤2
13	Urea + Ser	177	≤2
14	Urea + Lys	84	≤2
15	Urea + His	75	≤2
16	Urea + Glt	54	≤2
17	Urea + Gln	49	≤2
18	Urea + Gly	46	≤2
19	Urea + Asp	33	≤2
20	Urea + Asn	27	≤2
21	Urea + GABA	7	≤2
22	Urea + Arg	5	≤2
23	Urea + ornithine	4	≤2
24	Urea + Pro	≤2	≤2
25	Urea + allantoin	≤2	≤2
26	Urea + adenine	≤2	≤2
27	Urea + cytosine	≤2	≤2
28	Urea + allantoate	≤2	≤2

[a] Strains 23344c (*ura3*) and 30501c (*ssy1*Δ *ura3*) transformed with the CEN-based YCp*AGP1-lacZ* plasmid were grown on a minimal medium with the indicated compounds as the sole nitrogen source(s), each added at 5 mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were less than 20%.

TABLE 3. Agp1 is a broad-specificity amino acid permease[a]

Line no.	Amino acid	Uptake activity (nmol · min^{-1} · mg of protein^{-1})	
		*gap1*Δ	*gap1*Δ *agp1*Δ
1	Leucine	39	1
2	Isoleucine	40	5
3	Valine	22	4
4	Threonine	79	18
5	Phenylalanine	16	3
6	Tyrosine	5	1
7	Serine	130	50
8	Methionine	160	51
9	Alanine	54	20
10	Glutamine	103	38
11	Histidine	30	14
12	Asparagine	93	69
13	Glycine	19	10
14	Aspartate	122	111
15	Glutamate	41	41
16	Arginine	87	77
14	Proline	14	12
17	Tryptophan	2	2
18	Lysine	12	11

[a] Strains 30629c (*gap1*Δ *ura3*) and 30633c (*gap1*Δ *agp1*Δ ura3) were grown on minimal urea (1%) medium supplemented with citrulline (0.5%) to induce the synthesis of Agp1. ^{14}C-labeled amino acids were added at a 100 μM final concentration, and their initial uptake rates were determined in 2- or 4-min kinetic experiments.

normal in the *aro80*Δ mutant (36a). Thus, the presence of tryptophan leads to transcriptional induction of both the *AGP1* and *ARO9* genes, but the regulatory pathway involved in induction is apparently different for each gene. Expression of *AGP1-lacZ* and *ARO9-lacZ* was assayed in a *trp2^{fbr}* mutant, in which anthranilate synthase (Trp2p) is resistant to feedback inhibition by tryptophan (51). In keeping with previous reports (51, 82), this mutant growing on urea medium was found to accumulate endogenously synthesized tryptophan to levels that were at least 70-fold higher than in the wild-type (Table 4, lines 1 and 2). Expression of *AGP1-lacZ* and *ARO9-lacZ* was also assayed in the *gap1–92 agp1-1* strain, in which tryptophan is incorporated at a much lower rate than in the wild type (Fig. 6). In the *gap1–92 agp1-1* strain growing on urea medium, the intracellular tryptophan pool is as low as in the wild type; by 90 min after the addition of tryptophan (5 mM), it was about the same as in the *trp2^{fbr}* mutant growing on urea medium (Table 4, line 3). The results of β-galactosidase assays show that *ARO9-lacZ* is induced in cells that accumulate intracellular tryptophan. In contrast, *AGP1* remains insensitive to the intracellular accumulation of tryptophan (lines 1 and 2) and is specifically induced when tryptophan is added to the culture medium (line 3). This result clearly shows that *AGP1* is induced in response to extracellular rather than intracellular tryptophan. The role of Ssy1p is likely to detect the external amino acid and to activate a signal transduction pathway leading ultimately to transcriptional induction of the *AGP1* gene.

Induction of *AGP1* requires Grr1p. The Snf3p and Rgt2p proteins of the sugar transporter family act as sensors of external glucose concentration, sensors that can generate intracellular signals leading to glucose-regulated transcription (55, 67). The Grr1p protein plays a central role in the transduction of this glucose signal. This is shown, for instance, by the fact that *grr1* mutations relieve repression of many glucose-repressible genes (8, 28) and prevent induction by glucose of several *HXT* genes encoding glucose transporters (68). Grr1p is also involved in regulating the cell cycle, as it is required for degradation of the G_1 cyclins Cln1p and Cln2p (11). Grr1p is in fact a component of a ubiquitin-protein ligase complex (SCF^{Grr1}) (52) possibly involved in coupling nutrient availability to gene expression and cell cycle regulation (11, 54). In addition to impaired glucose signaling, *grr1* mutants display a number of other defects, including reduced uptake of aromatic amino acids (28) and leucine (69). These observations prompted us to test the role of Grr1p in Ssy1p-mediated induction of the *AGP1* gene. A mutant strain with a complete deletion of *GRR1* was isolated and used to monitor expression of the *AGP1-lacZ* gene. Induction of *AGP1-lacZ* by amino acids was abolished in the *grr1*Δ strain (Table 5). Hence, Grr1p is essential to the transduction of signals generated not only by the Snf3p and Rgt2p glucose sensors, but also by the Ssy1p amino acid sensor.

Induction of *AGP1* requires Uga35p, a transcription factor of the Cys_6-Zn_2 family. The *UGA35*(*DAL81*/*DURL*) gene encodes a transcription factor of the Cys_6-Zn_2 family, which is required for full induction of several nitrogen utilization genes (16, 21). These include the GABA-inducible genes involved in GABA utilization (*UGA1*, *UGA2*, and *UGA4*) (84, 85) and the allophanate-inducible genes involved in urea (*DUR1-2* and *DUR3*), allantoate (*DAL7*), and arginine (*CAR2*) utilization

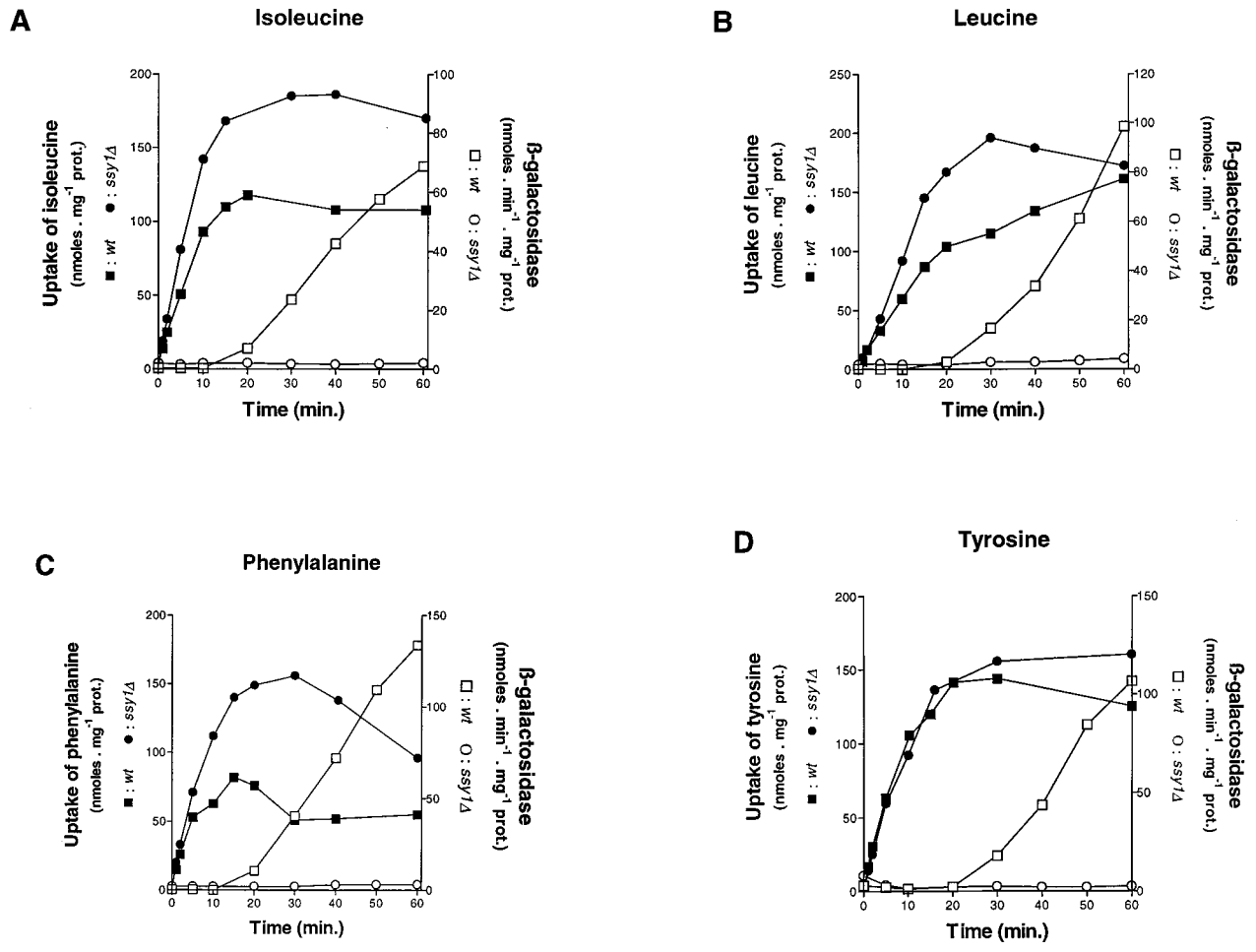

FIG. 5. Noninduction of the *AGP1* gene in the *ssy1*Δ mutant is not due to inducer exclusion. The time course of accumulation of ^{14}C-labeled amino acids (initial concentration, 0.1 mM) (solid symbols) and of the increase in β-galactosidase activity (open symbols) in strains 32501a (*ura3*) (squares) and 32501c (*ssy1*Δ *ura3*) (circles) transformed with the YCp*AGP1-lacZ* plasmid and initially growing on minimal medium with urea as the sole nitrogen source is shown.

(34, 40, 80). Induction of these genes requires a second transcription factor which, unlike Uga35p, is inducer specific (85). In the case of the GABA-inducible genes, the second factor is Uga3p (2); it is DurMp(Dal82p) in the case of allophanate-inducible genes (5, 40). In previous experiments, we observed that the *uga35* mutant displays resistance to β-2-thienylalanine (unpublished data). Although this resistance was not as pronounced as for the *ssy1*Δ mutant, we tested the role of Uga35p in the induction of *AGP1* (Table 5). The results clearly show that the induction of *AGP1-lacZ* is dramatically reduced in a *uga35*Δ mutant. It is unaltered, however, in *uga3* and *durM* mutants (data not shown). These results reinforce the previous conclusion that Uga35p is a pleiotropic factor involved in several transcriptional induction pathways (85). Uga35p does not seem, however, to specifically mediate Ssy1p-dependent induction, since it is also essential to allophanate-induced transcription (40, 85). Furthermore, Uga35p is required for induction of the *UGA4* gene by GABA (84), a regulation unaltered in the *ssy1*Δ mutant (data not shown).

TABLE 4. Transcription of *AGP1* is induced in response to external amino acids[a]

Line	Strain	Nitrogen source	Intracellular tryptophan (nmol · mg^{-1} [dry wt])	β-Galactosidase activity (nmol · min^{-1} · mg of protein^{-1})	
				AGP1-lacZ	*ARO9-lacZ*
1	Wild type	Urea	0.15	≤2	≤2
2	*trp2*^{fbr}	Urea	10	≤2	93
3	*gap1-92 agp1-1*	Urea	0.09	≤2	≤2
		Urea plus Trp (90 min)	12	560	80

[a] Strains 23346c (*ura3*), RE1 (*trp2*^{fbr} *ura3*), and 30622a (*gap1-92 agp1-1 ura3*) harboring the CEN-based plasmids YCp*AGP1-lacZ* or YCp*ARO9-lacZ* were grown on minimal medium with urea (5 mM) as the sole nitrogen source. Tryptophan was added at a 5 mM final concentration where indicated. The reported β-galactosidase activities are means of two independent experiments. The values of intracellular tryptophan concentrations are means of three experiments.

Ssy1p is required for induced transcription of at least five additional amino acid permease genes. To find additional genes that might display Ssy1p-dependent induction by amino acids, we purified total RNA from wild-type and *ssy1*Δ strains grown in the presence or absence of amino acids, treated it with DNase, and used it in RT-PCR experiments (see Materials and Methods). This approach enabled us to rapidly estimate the relative amounts of transcripts of several genes of the amino acid permease family. These included the inducible

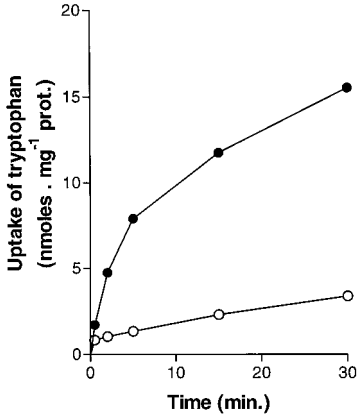

FIG. 6. The *gap1-92 agp1-1* strain is largely defective in tryptophan transport. The time course of ^{14}C-labeled tryptophan accumulation (initial external concentration, 5 mM) in strains 23344c (*ura3*) (●) and 30622a (*gap1-92 agp1-1 ura3*) (○) initially growing on minimal medium with urea (5 mM) as the sole nitrogen source is shown.

BAP2 gene proposed to encode a branched-chain amino acid permease (31), the *BAP3*(*PAP1*) gene encoding a close homologue of Bap2p (60), and several genes encoding probable amino acid permeases of unknown substrate specificity, namely, *ALP1* (78), *YFL055w* (63), *YPL274w* (17), *YBR132c* (27), and *YLL061w* (44). Some previously characterized genes, such as *MUP1* and *MUP3* (38), *TAT1* and *TAT2* (9, 75), and *GNP1* (90), were also included in this analysis because the reported experiments on expression of these genes were carried out in complex medium or in minimal medium containing amino acids to compensate for amino acid auxotrophies, i.e., under conditions where the expression of Ssy1p-responsive genes should appear constitutive. In the RT-PCR experiments

TABLE 5. Grr1p and Uga35p are essential to the transcriptional induction of the *AGP1* gene[a]

Line no.	Nitrogen sources	*AGP1-lacZ* β-galactosidase activity (nmol · min^{-1} · mg of protein^{-1})		
		Wild type	*grr1*Δ	*uga35*Δ
1	Urea	≤2	≤2	
2	Urea + Leu	638	≤2	
3	Urea + Ile	895	≤2	
4	Urea + Phe	960	≤2	
5	Urea + Tyr	724	≤2	
6	Urea + Met	744	≤2	
7	Urea + citrulline	284	≤2	
8	Proline	≤2		≤2
9	Proline + Leu	839		18
10	Proline + Ile	1,227		8
11	Proline + Phe	1,343		10
12	Proline + Tyr	791		5
13	Proline + Met	950		11
14	Proline + citrulline	235		≤2

[a] Strains 23344c (*ura3*), JA115 (*grr1*Δ *ura3*) and CD17 (*uga35*Δ *ura3*) transformed by the CEN-based YCp*AGP1-lacZ* plasmid were grown on minimal medium with the indicated compounds as the sole nitrogen source(s), each added at a 5 mM concentration. The *uga35*Δ was grown on proline instead of urea medium because of its inability to use urea as the sole nitrogen source. The reported β-galactosidase activities are means of two to three independent experiments. Variations were less than 20%.

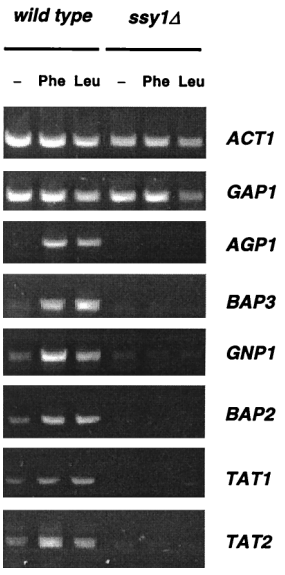

FIG. 7. The Ssy1p amino acid sensor affects expression of multiple amino acid permease genes. The results of RT-PCR analysis of the total RNA from strains 23344c (*ura3*) and 32501c (*ssy1*Δ *ura3*), with oligonucleotide primers specific to the actin gene (*ACT1*) and to several genes encoding amino acid permeases (see Materials and Methods), are shown. The cells were grown on minimal urea medium. At time zero, phenylalanine (5 mM) (Phe) or leucine (5 mM) (Leu) was added to part of the cultures, and the cells were collected after 90 min.

(Fig. 7), the amplified signal corresponding to the *AGP1* gene was undetectable in urea-grown cells but was very strong when phenylalanine or leucine was added to the cultures 90 min before they were harvested. As expected, the signal remained undetectable in the *ssy1*Δ mutant after amino acid addition. The *BAP3*, *TAT1*, *TAT2*, *BAP2*, and *GNP1* genes also displayed increased expression upon addition of leucine or phenylalanine, whereas their expression was barely detectable in the *ssy1*Δ mutant. Expression of *ALP1*, *YFL055w*, *YPL274w*, *YBR132c*, *YLL061w*, *MUP1*, and *MUP3* did not appear significantly different in wild-type and *ssy1*Δ cells (data not shown). As expected, the signal corresponding to the *GAP1* gene was roughly constant under all of the tested conditions. Although the results of these RT-PCR experiments must be confirmed by more-quantitative methods, they clearly show that several genes encoding amino acid permeases are under the positive control of the Ssy1p amino acid sensor.

DISCUSSION

In this study, we report the characterization in *S. cerevisiae* of Agp1p, a new broad-specificity amino acid permease. We show that transcription of the corresponding gene is induced in response to extracellular amino acids in a manner dependent on (i) an amino acid permease homologue acting as a sensor (Ssy1p), (ii) an F-box protein involved in glucose signaling and cell cycle control (Grr1p), and (iii) a transcription factor of the Cys_6-Zn_2 family involved in other nitrogen induction pathways (Uga35p) (Fig. 8). Furthermore, we provide evidence that this new nutritional signaling pathway influences the transcription of at least five other amino acid permease genes.

Agp1p, a broad-specificity amino acid permease in *S. cerevisiae*. Agp1p has the properties of a wide-specificity amino acid permease. Synthesis of this permease is associated with more-rapid uptake of many amino acids, including leucine, isoleucine, valine, threonine, phenylalanine, tyrosine, serine, methi-

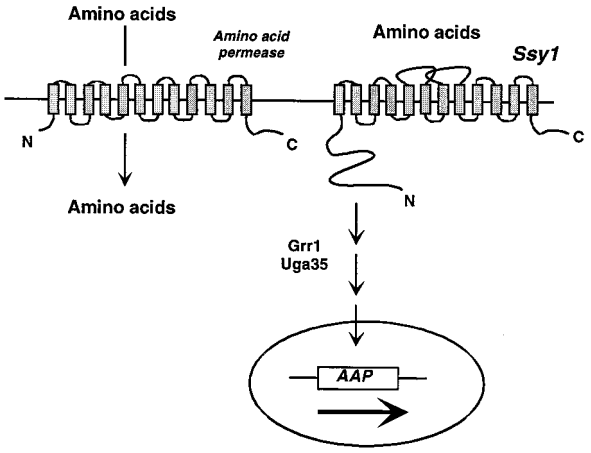

FIG. 8. Schematic presentation of the role of the permease-like amino acid sensor Ssy1p in the transcriptional regulation of amino acid permease genes in *S. cerevisiae*.

onine, alanine, glutamine, histidine, asparagine, and glycine (Table 3). Furthermore, growth tests suggest that Agp1p can also import tryptophan if present at a sufficiently high concentration. Agp1p appears as a relatively low-affinity permease. For instance, the K_m values for isoleucine, leucine, and tyrosine transport are in the 10^{-4} molar range and those for the transport of other amino acids, such as tryptophan, are probably much higher. Growth tests illustrate the importance of Agp1p in amino acid utilization: together with Gap1p, Agp1p is the main amino acid permease ensuring growth on isoleucine, leucine, phenylalanine, tyrosine, and tryptophan as the sole nitrogen source and also contributes significantly to the utilization of valine, methionine, and threonine (1 mM). The absence of any clear growth defect of the *gap1 agp1* strain when grown on higher concentrations of some of these amino acids (10 mM) or on other substrates of Agp1p such as glutamine, asparagine, serine, and alanine is likely due to the activity of other amino acid permeases which can compensate for the lack of Agp1p. Interestingly, our growth tests failed to show any contribution of Bap2p, defined as the major branched-chain amino acid permease (31), in the utilization of leucine and isoleucine (1 mM) as the sole nitrogen source (Fig. 3). Similarly, Tat1p, defined as a major tyrosine permease in yeast cells (75), is unable to ensure tyrosine utilization at a 1 to 10 mM final concentration (Fig. 3). These observations raise questions as to the actual physiological function of these permeases.

As this paper was being written, it was reported by Schreve et al. (76) that the primary substrates of Agp1p are asparagine (K_m = 0.29 mM) and glutamine (K_m = 0.79 mM), and the permease was named Agp1p to reflect this substrate specificity. It was further suggested that Agp1p mediates the uptake of other amino acids but that the affinity of the permease for these amino acids is lower than for asparagine and glutamine (76). These data are hardly consistent with the K_m values of Agp1p for leucine (0.16 mM), isoleucine (0.6 mM), and phenylalanine (0.6 mM) transport. Rather, the affinity of Agp1p appears to be in the same range for many amino acids. Schreve et al. also reported that the pattern of *AGP1* expression according to the nitrogen source is very similar to that of the *GAP1* gene: the reason why the induction by amino acids was not revealed in these experiments is probably because the leucine was systematically added to the growth medium in order to compensate for a *leu2* auxotrophy.

Agp1p also mediates methionine uptake (Table 3). It was previously reported that methionine is transported in yeast cells by at least three different permeases: the high-affinity permease (K_m = 0.013 mM) encoded by the *MUP1* gene, a low-affinity permease (K_m = 0.2 mM) whose gene (*MUP2*) remained uncharacterized, and a very-low-affinity permease (K_m = 1 mM) encoded by the *MUP3* gene (38). The low-affinity permease is probably Agp1p: its activity was measured under growth conditions leading to the induction of *AGP1*, it displays a broad specificity range, and methionine transport mediated by this permease is inhibited by leucine with an apparent K_i value (0.30 mM) (38) very close to the K_m value of Agp1 for leucine.

***AGP1* is induced by multiple amino acids: role of a permease-like sensor of external amino acids.** Transcription of the *AGP1* gene is induced by many amino acids, a notable exception being proline (Table 2). Induction levels vary considerably according to the amino acid tested, and most inducing amino acids are also substrates of the Agp1p permease. Induction of *AGP1* is abolished in mutants lacking Ssy1p, a protein of the amino acid permease family acting as a sensor of extracellular amino acids. The first evidence for a role of this protein in amino acid sensing was recently provided (24). It was found that induction by leucine (0.23 mM) of the *BAP2*, *BAP3*, and *TAT1* genes encoding amino acid permeases (31, 60, 75) and of the *PTR2* gene encoding a peptide transporter (71) is abolished in the *ssy1* strain. Induction of *BAP2* by L- or D-leucine still occurs in a mutant strain largely deficient in L- and D-leucine uptake, leading to the suggestion that Ssy1p acts as a sensor of external leucine (24). Using RT-PCR, we have confirmed that expression of *BAP2*, *TAT1*, and *BAP3* is under the positive control of Ssy1p and found that the same is true of *AGP1*, *GNP1* encoding a glutamine permease (90), and *TAT2* encoding a tryptophan permease (75). These genes seem, in fact, to be induced by other amino acids besides leucine (Table 2; Fig. 7, and unpublished data); this means that Ssy1p is probably involved in the transcriptional induction of several permease genes in response to various amino acids. Our data on the *AGP1* gene show that permease genes under the control of Ssy1p are transcriptionally induced in response to extracellular rather than intracellular amino acids. For instance, *AGP1* remains unexpressed in a $trp2^{fbr}$ mutant endogenously accumulating tryptophan to relatively high levels. It is, however, markedly induced after the addition of tryptophan to a mutant strain largely defective in tryptophan uptake even though the intracellular tryptophan pool is about the same as in the $trp2^{fbr}$ strain failing to induce *AGP1*. We thus propose that the Ssy1p permease homologue detects external amino acids and activates, in turn, a transduction pathway leading ultimately to transcriptional activation of several permease genes.

The Ssy1p protein displays structural features that clearly distinguish it from Agp1p, Gap1p, and the other members of the amino acid permease family. In particular, its hydrophilic N terminus is much larger, as are several regions connecting TM domains and predicted to be extracellular (Fig. 2). These features are reminiscent of those displayed by the Snf3p and Rgt2p glucose sensors, which differ from the other members of the sugar transporter family by their unusually long cytoplasmic C-terminal domain (55, 66, 67). These domains have been shown to be essential to the role of Snf3p and Rgt2p as glucose sensors (66). Furthermore, grafting these domains onto the C terminus of Hxt1p confers to this glucose transporter the properties of a glucose sensor (66). The large N-terminal domain of Ssy1p likely plays an important role in generating the amino acid signal and could, for instance, mediate interaction with another protein. A candidate protein is the *PTR3* gene product

(10), a hydrophilic 76-kDa protein originally discovered as a factor essential to the induction of the *PTR2* gene in response to amino acids (37). Mutants affected in this gene were subsequently and independently isolated as *shr6* (49), *ssy3* (46), and *apf3* mutants (13a). Although the exact function of Ptr3p remains undetermined, the phenotypes of the *ptr3*/*apf3*/*ssy3*/*shr6* and *ssy1* mutants appear indistinguishable (13a, 46, 49).

The Ssy1p amino acid sensor might activate a signal transduction pathway upon binding of amino acids without translocating them across the plasma membrane. As such, Ssy1p would act as a receptor. Alternatively, Ssy1p-mediated transport of amino acids could be essential to the protein's signaling function. This hypothesis, however, implies that the transport capacity of Ssy1p should be very low, since a *gap1*Δ *agp1*Δ *SSY1*$^+$ strain is unable to grow on several amino acids as the sole nitrogen source (1 mM) (Fig. 3), even though Ssy1p effectively transmits signals in response to these amino acids.

Grr1p: an F-box protein involved in glucose signaling, amino acid signaling, and cell cycle control. The Grr1p protein plays a central role in transducing signals generated by the Snf3p and Rgt2p glucose sensors (54) and in degrading G_1 cyclins (11). This F-box protein is a component of a so-called SCF complex (for Skp1–Cullin–F-box) belonging to a novel class of ubiquitin-protein ligases (E3) that probably exist in organisms ranging from yeast to humans (26, 52). A typical SCF complex consists of (i) Cdc53p, a protein functioning as a scaffold subunit and belonging to a large, evolutionarily conserved family of proteins called cullins (62, 70, 88); (ii) an F-box protein, involved in selecting which potential targets are to be ubiquitinated (70, 77); and (iii) Skp1p, which forms a bridge between Cdc53p and one of several F-box proteins (Grr1p, Cdc4p, and Met30p) (7). SCF complexes function in combination with an E2 enzyme catalyzing transfer of ubiquitin to the target protein. For instance, an SCF complex containing Grr1p as the F-box protein promotes ubiquitination of G_1 cyclins in conjunction with the E2 enzyme Cdc34p (22, 81, 89). On the other hand, transduction of the glucose signal generated by Snf3p leading to induction of the *HXT1* gene requires Grr1p, Cdc53p, and Skp1p, but Cdc34p is not needed, suggesting that another E2 enzyme is involved (54).

In this study, we show that Grr1p is also essential to Ssy1p-mediated induction of the *AGP1* gene, thus extending the role of this F-box protein to an additional regulatory pathway. It seems reasonable, therefore, to speculate that transduction of the signal generated by the Ssy1p sensor involves an SCF complex with Grr1p as the F-box–protein subunit. The complex could be involved, for instance, in ubiquitinating a down-regulator of Ssy1p-regulated genes in response to external amino acids. Regarding possible targets of this putative SCF^{Grr1} complex, it is noteworthy that *PTR2*, a peptide transporter gene whose induction by amino acids is Ssy1p dependent (24), is under the negative control of Cup9p (50), a short-lived homeodomain protein ($t_{1/2}$, ~5 min) whose degradation involves the ubiquitin pathway (18). The Ptr1p-Ubr1p protein proposed to act as a ubiquitin-protein ligase (83) is essential both to Cup9p degradation (18) and to *PTR2* induction (1, 37). Furthermore, the E2 enzymes encoded by the *UBC2* and *UBC4* genes appear essential to Cup9 degradation (18). We are currently conducting experiments to test the roles of Cup9p, Ptr1p-Ubr1p, Ubc2p, Ubc4p and of the SCF complex components Skp1p, Cdc53p, and Cdc34p in the expression of amino acid and peptide permease genes under Ssy1p control.

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